ABSTRACT
This protocol details methods for 3-day Antigen Presentation Assay.

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PROTOCOL CITATION
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KEYWORDS
antigen, presentation, Ag

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Nov 11, 2020  Liz Brydon  Protocols.io
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MATERIALS TEXT

REAGENTS
- LPS/IFNg (including controls)
- RPMI media
- PFA
- PBS
- Wash buffer: DMEM with 0.1M glycine + 10% iFBS
- Stock peptide
- 1x lysis buffer (stock = 5x, with triton, pH = 7.8) in dH2O
- 1M DTT
- CPRG buffer
- Water
- CPRG

CONSUMABLES
- 96-well plates
- 50ml conical tubes

EQUIPMENT
- Incubator: 37°C with 5% CO2
- Centrifuge
- Cell counter
- Plate reader

SAFETY WARNINGS
Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

Day 1

1. Prepare a 96 well plate:
   - Label appropriately (experimental condition – LPS/IFNg controls, infection controls, etc.)
   - Duplicate labelled wells for the peptide control

2. Scrape RAW cells and resuspend (pipette up and down 8x).

3. Count RAW cells and adjust concentration to **0.75 million cells/ml**.

4. Add **200 µl RAW cells** to each well.

5. Add LPS/IFNg accordingly.

6. Incubate for **24:00:00** at **37 °C with 5% CO2**.

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Day 2

7 Prior to beginning, check the cell’s media (red/orange = good, yellow = bad).

8 Prepare **1%** (v/v) PFA in PBS at **Room temperature** (**pH 7.4**).

9 Discard supernatant in the 96 well plate and add **50 µl** 1% PFA.

9.1 Incubate at **Room temperature** for **00:10:00**.

10 Prepare 2E2 cells while RAW cells are in 1% PFA:

10.1 Pour flask into 50 ml conical and centrifuge at **1500 rpm, 00:03:00**.

To keep more 2E2 cells growing, add **50 mL RPMI media** back into their flask and incubate at **37 °C, 5% CO2**.

10.2 Count 2E2 cells and adjust concentration to **0.4 million cells/ml**.

11 Add **200 µl wash buffer** to each well in the 96 well plate and discard immediately.

Wash buffer = DMEM with 0.1M glycine, + 10% iFBS.

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Repeat 2x more: Add **200 µl wash buffer** to each well in the 96 well plate and discard immediately. *(Wash 1/2)*

### 11.2 Repeat 1x more: Add **200 µl wash buffer** to each well in the 96 well plate and discard immediately. *(Wash 2/2)*

**Add 250 µl 2E2 cells** per well in ½ of the samples (the non-peptide ones). Add an extra row of 2E2 cells alone for a negative control.

### 12

Dilute stock peptide (1 µg/ml) **5000 fold** in the 2E2 cells (final concentration: **0.2 nanogram per milliliter (ng/mL)**).

**Add 250 µl peptide + 2E2 cell solution** to the remaining wells.

### 13

Incubate at **37 °C, 5% CO2** for **16:00:00 (NO LONGER)**.

#### Day 3

**Prepare 1x lysis buffer (stock = 5x, with triton, pH 7.8) in dH₂O.**

#### 16.1 **Add 30 µl 1M stock of DTT in 10 mL lysis buffer.**

**Prepare CPRG buffer (pH 7.8).**

### 17

Centrifuge 96 well plate at **2200 rpm, 00:01:00**.
Add 50 µl lysis buffer to all wells.

19.1 Incubate at Room temperature for 00:05:00 (up to 20 minutes MAX).

20 Prepare CPRG (recipe below = for each well)
- 150 µl CPRG buffer
- 20.2 µl water
- 0.046 mg CPRG

21 When lysis is done, add 170 µl CPRG solution / well.

21.1 Incubate either at Room temperature or 37°C (37 speeds up reaction to about ~ 20 minutes for peptide samples).

22 Transfer 150 µl colored solution to a new plate.

Take care not to transfer debris or make bubbles.

23 Take reading at 595 nm or 570 nm.

* To stop reaction to leave overnight, incubate at 4 °C.